REMARKS/ARGUMENTS

Status of the Claims

Claims 1 to 3, 7, 8, 12, 13 and 47 to 50 were previously undergoing examination on the merits. Claims 1, 47, 49, and 50 are amended herein. Claim 51 to 58 are new. Claims 9, 10 and 19 to 46 are canceled without prejudice. Claims 4 to 6, 11, and 14 to 18 stand withdrawn from consideration. No amendments are an acquiescence to a position adopted by the Office. After entry of the amendments, claims 1 to 3, 7, 8, 12, 13 and 47 to 58 will be pending.

Applicants thank the Examiner for reconsidering and withdrawing the objections to claims 2, 7, and 8 and the rejection of claims 1 to 3, 7, 8. 12, 13, and 46 to 50 under 35 U.S.C.§ 103(a) over Wels et al. and Pastan et al.

Claims 1 to 3, 7 to 8, 12 to 13 and 47 to 50 stand rejected as not conforming with the Written Description requirement of 35 U.S.C. §112, first paragraph.

Claims 1 to 3, 7, 8 12, 13, and 47 to 50 stand rejected for alleged indefiniteness under 35 U.S.C. §112, second paragraph.

Claim 47 stand objected to as depending from a canceled claim.

Claims 1, 3, 7, 12 to 13, and 47 to 50 stand rejected for alleged indefiniteness under 35 U.S.C. §112, first paragraph on various grounds

Claims 49 and 50 stand rejected for alleged indefiniteness under 35 U.S.C. §112, second paragraph with respect to the recital of "the cell."

Claims 1, 7 to 8, 12, and 47 to 50 stand rejected under 35 U.S.C. §102(e) as allegedly anticipated by Cardy et al. (U.S. Patent Publication No. 2002/0106370).

Applicants further respond to these rejections below.

Amendments to the Claims

Claim 1 was amended to recite an "epithelial cell surface receptor on the apical surface of a mucosal membrane." Support for such subject matter can be found, *inter alia,* in the Section entitled "Cell-based Studies" beginning at the bottom of page 52. Claim 1 was further amended to recite a cysteine-cysteine loop. Support for such subject matter can be found,

inter alia, in the specification at p. 23, first three lines. The remainder of the amendments are supported by the previous version of the claim.

Claim 47 was amended to correct its dependency from a canceled intervening claim to the base claim. Support for this subject matter is found, *inter alia,* in the previous version of the claim.

Claims 49 and 50 have been amended to recite "mammal is" in place of "cell is from." Support for this subject matter is found, *inter alia,* in the previous version of the claims.

New claim 51 recites "the translocation domain comprises an amino acid sequence consisting essentially of the PE amino acid sequence (SEQ ID NO:2) from amino acid position 280 to amino acid position 344 thereof." Support for such subject matter can be found, *inter alia,* in original claim 1.

Support for new claims 52 to 54 respectively can be found, *inter alia,* in previous claims 1, 2, and 3.

New claims 55 and 56 set forth that the cysteine-cysteine loop of the pathogen is located within PE domain Ib in place of amino acid residues 372 to 379 thereof. Support for this subject matter is found, *inter alia,* in the specification at p. 28, lines 7 to 11.

Support for new claims 57 and 58 is found, *inter alia,* in previous claims 2 and 3.

In view of the above, the Applicants believe the amendments to the claims add no new matter and respectfully request their entry.

Response to the Rejection based upon the Alleged Disclosure of Essential Subject Matter by Reference

The Examiner alleges that the specification fails to recite essential matter related to the amino acid sequence of the translocation domain at positions 339, 343, 341, or 344 of SEQ ID NO:2. Applicants respectfully disagree. In particular, the specification does disclose at p. 27, lines 21 to 22 that the amino acids at positions 339 and 343 appear to be necessary for translocation.

In pertinent part, the base claim recites:

> a translocation domain having an amino acid sequence at least 95% identical to the sequence of *Pseudomonas* exotoxin A (PE)

(SEQ ID NO:2) from amino acid position 280 to amino acid position 344 thereof and wherein the domain is capable of effecting translocation to the cytosol of a cell

In view of the above recital, Applicants submit that the essential subject matter pertaining to the domain II sequence variants is that they be at least 95% identical to the specified sequence and that the variants must be capable of effecting translocation to the cytosol of a cell. Such subject matter is disclosed in the specification at p. 26 and 27.

While the Office alleged that the specification does not disclose that amino acids at positions 339 and 343 appear to be necessary for translocation, this disclosure as noted above can be found in the specification at p. 27, lines 21 to 22. To the extent that the amino acids at positions 339 and 343 are necessary for translocation, the functional recital fully embraces such subject matter and such subject does need not to be further elaborated. Accordingly, the essential subject matter pertinent to a functional translocation domain is disclosed in the specification.

The Office was also concerned that the specification did not disclose, other than by incorporating the Siegall et al. reference, that amino acids at positions 341 and 344 can be mutated. In this regard, the Applicants note that not all the subject matter which may be embraced by a claim need be disclosed in the specification. There is simply no obligation to set forth all the modes for practicing an invention in a specification. Only the best mode must be disclosed and that only if the Applicants have one in mind. Applicants have not set forth that those particular variants having altered sequences at position 341 and 344 represented their best mode for practicing the claimed invention. Accordingly, the description of such alternative embodiments are not essential subject matter and need not be expressly set forth in the specification.

Response to the Rejection of Claims 1 to 3, 7 to 8, 12 to 13 and 47 to 50 as Allegedly not in Conformity with the Written Description requirement of 35 U.S.C. §112, first paragraph.

The Office contends that the specification does not convey to one of ordinary skill in the art that the Applicants were in possession of the claimed subject matter at the time of filing. As amended, the base claim now sets forth in pertinent part:

> a translocation domain having an amino acid sequence at least 95% identical to the sequence of *Pseudomonas* exotoxin A (PE) (SEQ ID NO:2) from amino acid position 280 to amino acid position 344 thereof and wherein the domain is capable of effecting translocation to the cytosol of the cell;

The above language embraces translocation domains having the amino acid sequence of SEQ ID NO:2 from amino acid position 280 to amino acid position 344 and variants thereof having at least 95% sequence identity from amino acid position 280 to amino acid position 344 of SEQ ID NO:2. The Office contends that the specification does not provide adequate written description commensurate with the breadth of the claim. Applicants respectfully disagree.

Standard of Review

The Written Description requirement can be met by showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics ...., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. *Enzo Biochem. v. Gene Probe, Inc.* 296 F.3d (Fed. Cir. 2002) at 1324, 63 USPQ2d at 1613.

Here (*see,* specification Section C: Translocation Domain on pages 27 and 27), the Applicants have disclosed the amino acid sequence of the translocation domain of PE II and sets forth the portion thereof (from amino acid positions 280 to 344 of domain II) for the translocation function. The specification also discloses that the domain can be engineered with substitutions so long as translocation activity is retained. The specification states that amino acids at positions 339 and 343 appear to be necessary for translocation. The specification discloses the use of several ntPE chimeras in which the amino acid sequence of the translocation

domain is identical to the sequence of the relevant portion of SEQ ID NO:2. The procedures for making variants of SEQ ID NO: 2 are conventional in the art and were well established at the time of filing. The specification evidences such in citing the Siegall et al., *Biochemistry* 30(29):7154-9 (1991) reference and several patents to Pastan et al. (e.g., U.S. Patents Nos. 5,602,095; 5,512,658, and 5,458,878; *see*, page 22 last three lines). In addition, several assays for screening for translocation activity are also described (*see,* page 32 of the specification, Subsection 2, Translocation to the Cytosol) which can be used to determine whether a variant has the recited activity.

Applicants further refer the Examiner to the enclosed reference co-authored by the inventors (Kasturi et al., *J. Biological Chemistry* 2676(32):23427-23433 (1992). Kasturi et al. used alanine scanning mutagenesis to identify the surface amino acids on Domain II which were required for cytotoxicity, proper folding, and secretion into periplasm). They made about 19 substitutions with alanine at 19 positions in Exotoxin A domain II from positions 280 to 344 and found 3 such substitutions which rendered the Exotoxin A inactive and 16 which retained at least some activity including four substitutions being as active or more active than the native Exotoxin A (*see*, Table VI at p. 23433 of Kasturi et al.). Accordingly, suitable functional mutations of the translocation domain, and methods of obtaining them, were well known in the prior art.

While the Enzo standard can be difficult to translate into practice, the Office has provided Written Guidelines for assessing how well a disclosure meets the Written Description requirement of 35 U.S.C. §112, first paragraph (*see*, Revised Interim Written Guidelines Training Materials, Example 14, pages 53 to 55).

The instant case is almost exactly on point with respect to Example 14 of the Office's Written Description Guidelines which specifically concern a hypothetical invention claiming a protein having at least 95% sequence homology to the disclosed sequence of the solely exemplified protein and the biological activity of the disclosed protein. In conclusion, the guidelines recited:

> A review of the full content of the specification indicates that a protein having SEQ ID NO: 3 or variants having 95% identity to SEQ ID NO: 3 and having catalytic activity are

> essential to the operation of the claimed invention. The procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity. Moreover, procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art. A review of the claim indicates that variants of SEQ ID NO: 3 include but are not limited to those variants of SEQ ID NO: 3 with substitutions, deletions, insertions and additions; but all variants must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO: 3.
>
> Additionally, the claim is drawn to a protein which **comprises** SEQ ID NO: 3 or a variant thereof that has 95% identity to SEQ ID NO: 3. In other words, the protein claimed may be larger than SEQ ID NO: 3 or its variant with 95% identity to SEQ ID NO: 3. It should be noted that "having" is open language, equivalent to "comprising".The claim has two different generic embodiments, the first being a protein which comprises SEQ ID NO: 3 and the second being variants of SEQ ID NO: 3. There is a single species disclosed, that species being SEQ ID NO: 3. A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious. There is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.
>
> **Conclusion:** The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.

Applicants note that the open-ended nature of the language of the hypothetical claim was expressly noted in bold by the Written Guidelines and was not set forth as a grounds for rejection. Thus, based upon the Applicants' disclosure and the Office's own guidelines, one of ordinary skill would certainly understand that the Applicants were in possession of the invention as claimed at the time of filing.

Accordingly, the Applicants request that any rejections based upon the above allegations be reconsidered and withdrawn.

Response to the Rejection of Claims 1 to 3, 7 to 8, 12 to 13, and 47 to 50 as allegedly indefinite with respect to the recital of "cysteine-to cysteine bonded loop."

The Office alleged the recital of a "cysteine-to cysteine bonded loop" in the previous version of the base claim to be ambiguous as possibly indicating a loop of just two amino acids in length and therefore inconsistent with the stated length of the epitope presenting domain as being from 5 to 350 amino acids.

Without acquiescing to the position of the Office, the Applicants have amended the base claim to recite:

> an epitope presenting domain of between 5 and 350 amino acids in length and consisting essentially of one cysteine-cysteine loop of a pathogen wherein the loop encodes an epitope of the pathogen and wherein the epitope is non-native to PE domain Ib

The term "cysteine-cysteine loop" is defined in the specification (at page 23, first two lines) as an amino acid sequence bordered by two disulfide-bonded cysteine residues. Accordingly, the Applicants respectfully request that the above rejection be reconsidered and withdrawn.

Response to the Rejection/Objection of Claim 47.

The Applicants have amended the claim to depend from claim 1. Accordingly, the Applicants respectfully request that the above rejection be reconsidered and withdrawn.

Response to the Rejection of Claims 49 and 50 for indefiniteness under 35 U.S.C. §112, first paragraph with respect to the recital of "the cell."

Claims 49 and 40 have been amended to recite "mammal is" in place of "cell is from." The term mammal finds antecedent basis in the base claim. Accordingly, Applicants respectfully request that the above rejection be reconsidered and withdrawn.

Response to the Rejection of Claims 1, 7 to 8, 12, and 47 to 50 for Alleged Anticipation under 35 U.S.C. §102(e) by Cardy et al. (U.S. Application Publication No. 2002/0106370.

As a threshold matter, Applicants note that the base claim has been amended to set forth

> a cell recognition domain of between 10 and 1500 amino acids that binds to an epithelial cell surface receptor on the apical surface of a mucosal membrane of a mammal;

Applicants note that Cardy et al. do not disclose or suggest a cell recognition domain which binds to an epithelial cell surface receptor on the apical surface of a mucosal membrane of a mammal.

With regard to new independent claim 56, Cardy et al. do not disclose locating the cysteine-cysteine loop of a pathogen in place of amino acid residues 372 to 379, inclusive, of SEQ ID NO:2.

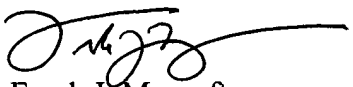
Accordingly, Applicants respectfully request that the above rejection be reconsidered and withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



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Alanine Scanning Mutagenesis Identifies Surface Amino Acids on Domain II of *Pseudomonas* Exotoxin Required for Cytotoxicity, Proper Folding, and Secretion into Periplasm*

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***Pseudomonas* exotoxin A (PE) is a single polypeptide chain that contains 613 amino acids and is arranged into three major structural domains. Domain Ia is responsible for cell recognition, domain II for translocation of PE across the membrane, and domain III for ADP-ribosylation of elongation factor 2. Recombinant PE can be produced in *Escherichia coli* and is efficiently secreted into the periplasm when an OmpA signal sequence is present. To investigate the role of the amino acids located on the surface of domain II in the action of the toxin against mammalian cells, we substituted alanine for each of the 27 surface amino acids present in domain II. Surprisingly, all 27 mutant proteins had some alteration in cytotoxicity when tested on human A431 or MCF7 cells or mouse L929 cells. Native PE has a compact structure and therefore is relatively protease resistant and very little ADP-ribosylation activity is detected in the absence of the denaturing agents like urea and dithiothreitol. Several of the mutations resulted in altered protease sensitivity of the toxin. Seven of the mutant molecules exhibited ADP-ribosylation activity without urea and dithiothreitol, indicating they are partially unfolded. Out of these seven mutants, six had increased cytotoxic activity on at least one of the target cell lines and the other retained its native cytotoxic potency.**

Pseudomonas exotoxin A (PE)[1] is a 66,000 molecular weight protein that is secreted by *Pseudomonas aeruginosa*. The toxin enters eukaryotic cells by receptor-mediated endocytosis and then is translocated to the cytosol where it catalyzes the irreversible inactivation of elongation factor 2 which arrests protein synthesis and results in cell death. The three-dimensional structure of PE has been solved by Allured *et al.* (1) who showed that the protein crystal is composed of three prominent domains. Domain Ia is composed of residues 1–252, domain II of residues 253–364, domain Ib is a minor domain consisting of residues 365–399, and domain III of residues 405–613.

We have been interested in defining functions of each domain and identifying the residues in each domain that are necessary for the cytotoxic action of PE. For this purpose DNA encoding each of these domains was expressed in *Escherichia coli*, and their functions are analyzed. These studies have shown that domain Ia is responsible for cell recognition. Deletion of domain Ia abolishes cell binding and decreases its cytotoxicity against Swiss 3T3 cells by greater than 1000-fold (2). Lysine 57 is one of the amino acids that is required for cell binding; mutations at position 57 reduce cytotoxicity about 100-fold, and such mutants fail to compete for binding and for the cytotoxic action of native PE (3). Domain II is involved in translocation of the toxin across the membrane. Deleting a major portion of domain II reduces the toxicity of PE without diminishing cell binding or ADP-ribosylating activity (2). Furthermore, domain II can promote the translocation of sequences placed in the position of domain III (4, 5). Domain III catalyzes the ADP-ribosylation of elongation factor 2 which results in protein synthesis inhibition and cell death. Residue 553 is important since it interacts with NAD (6). A mutation which changes Glu^{553} to Asp greatly reduces ADP-ribosylation activity and deletion of Glu^{553} abolishes the enzyme activity completely (7). By mutational analysis Chaudhary *et al.* (8) found that the last five amino acids of PE (REDLK) are critical for its cytotoxic activity although unrelated to its ADP-ribosylation activity.

To study the function of residues in domain II which might be involved in the translocation of PE and chimeric toxins derived from PE, Siegall *et al.* (9) made a series of deletion mutants in domain II. Removal of the amino-terminal portion of domain II (residues 253–303) or an internal portion of domain II (residues 337–362) dramatically reduced cytotoxic activity. In all these cases, ADP-ribosylation activity was fully retained. The fact that these molecules had an intact binding domain and full ADP-ribosylation activity indicates that domain II is required for a step after binding and internalization and prior to ADP-ribosylation. Using radiolabeled PE, Ogata *et al.* (10) analyzed the processing of PE in mammalian cells and found that PE is cleaved within a region of domain II that is bounded by a disulfide bridge connecting cysteine 265 and 287. The exact cleavage site is between Arg^{279} and Gly^{280}.[2] Proteolysis is followed by reduction of the disulfide bond (268–287) and the 37-kDa fragment (COOH-terminal) that is released is ultimately delivered to the cytosol where it ADP-ribosylates elongation factor 2 and arrests protein synthesis. These data provide a general outline of the requirement of portions of domain II for toxicity, but the role of specific amino acids in domain II is not known.

The three-dimensional structure of PE reveals that domain

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[1] The abbreviations used are: PE, *Pseudomonas* exotoxin; DTT, dithiothreitol; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

[2] Ogata, M., Fryling, C. M., Pastan, I., and FitzGerald, D., (1992) *J. Biol. Chem.*, in press.

II is composed of six α-helices and contains many surface residues which could participate in the functions carried out by domain II. To study the role of these amino acids in the action of PE, we replaced each of the 27 amino acids on the surface of domain II with alanine. Alanine was chosen as a replacement residue because it eliminates the side chain beyond the β-carbon yet does not alter the main chain conformation, nor does it impose extreme electrostatic or steric effects. Furthermore, alanine is the most abundant amino acid in proteins and is found in both buried and exposed positions and in all varieties of secondary structure (11). Our results indicate that all the surface amino acids in domain II contribute to its activity and changes in these amino acids affect cytotoxic activity as well as the structure of PE.

MATERIALS AND METHODS

HB101 (SE) from Bethesda Research Laboratories was used for the propagation of the plasmids. pVC45 f+T that encodes a secreted form of native PE has been described previously (12). Oligonucleotide-directed mutagenesis was carried out by a modification of the method of Kunkel (13). Oligonucleotides with one mutation each, with a new restriction site introduced or a unique restriction site removed, were synthesized using an Applied Biosystems DNA synthesizer. Single-stranded DNA prepared from first cycle phage in uridine-containing medium was used as a template for *in vitro* mutagenesis (12, 14). Several colonies were screened for the presence or absence of unique restriction sites and the mutations were confirmed by DNA sequencing. Mutant plasmids were directly expressed in the T7 promoter based expression system in BL21 as previously described (15) unless otherwise stated. The list of all the constructs, the position of the amino acid that was changed to alanine is replaced, the unique restriction sites introduced/removed, and the summary of expression in the BL21 strain of *E. coli* are given in Table I.

Purification—Mutant proteins extracted from the periplasm in low ionic strength buffer were bound to QMA cartridges (water). To elute contaminating proteins, the cartridges were washed with 5 ml each of 20 mM Tris, pH 7.4; 20 mM Tris, 100 mM NaCl, pH 7.4; 20 mM Tris, 150 mM NaCl, pH 7.4. PE was eluted in 20 mM Tris, 200 mM NaCl, pH 7.4. One-ml fractions were collected and frozen at −80 °C.

Gel Electrophoresis and Immunoblotting—The individual fractions eluted in 20 mM Tris, 200 mM NaCl were run on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. The fractions showing a single major band corresponding to 66 kDa were pooled, concentrated on Centricon 30 microconcentrator, and frozen at −80 °C. For immunoblotting, samples were transferred from gels to nitrocellulose paper and antibodies to PE (2) and Vecta Stain kits (Vector Laboratories, Burlingame, CA) were used to visualize PE and its mutant forms.

Protein Synthesis Inhibition Assay—The cytotoxic activity of PE and its mutant forms was determined by assaying the inhibition of protein synthesis using L929 (mouse fibroblasts), A431 (epidermoid carcinoma), and MCF7 (breast cancer) cell lines. Protein synthesis was assayed by determining the incorporation of [^{3}H]leucine in trichloroacetic acid precipitates of the cells (3, 16, 17).

ADP-ribosylation Assay (in Vitro Enzyme Activity)—ADP-ribosylation activity of protein samples were measured by following the procedure of Collier and Kandel (18) using a wheat germ extract enriched in elongation factor 2. The assay was carried out with or without activation by incubation with 4 M urea and 40 mM DTT before the assay.

Assay of Protease Sensitivity of PE and All of Its Mutants—Trypsin and chymotrypsin were used to compare the relative sensitivity of PE and its mutants to proteolytic cleavage. In general, 10 μg of protein was incubated at room temperature at pH 7.5 with different amounts of protease in a volume of 50 μl. The time of incubation was 1 h for trypsin and 2 h for chymotrypsin. At the end of the incubation, proteins were analyzed on 12.5% SDS-polyacrylamide gels and stained with Coomassie Blue.

RESULTS

The amino acids exposed on the surface of PE were identified using an integrative program for computing the accessible surface area and volume by the Lee and Richard's algorithm (19). All the amino acids in domain II of PE whose accessible surface area is ≥90 Å^2 were considered the most exposed, but we also mutated several others with surface areas below 90 Å^2. The list of amino acids is given in Table I. Oligonucleotide-directed mutagenesis was performed to replace these residues with alanine. The DNA sequence of each mutant clone was determined to be certain that the correct mutation was observed.

Expression and Purification of PE and Its Mutants—Plasmids encoding each of these mutant proteins were expressed in *E. coli* BL21 (λDE3), and most were secreted into the periplasm (as is wild type PE) because of the presence of an OmpA signal sequence (17). However, mutants H266A, R313A, and T355A were not secreted and remained within the cells. To obtain soluble protein for analytical studies, these mutant proteins were expressed in a BL21 (λDE3) strain containing a plasmid encoding the GroE complex (19) which resulted in a portion of each of the proteins being secreted into the periplasm although the yield was low compared with native PE (data not shown). Each mutant protein was purified from the periplasm to greater than 95% homogeneity using QMA SEP-PAK cartridges (Fig. 1). Mutant R352A did not express protein.

Cytotoxic Activity of Mutant Proteins—To determine the

TABLE I

The list of all the constructs, the position, and native amino acid in wild type PE at which alanine is replaced

The unique restriction site is introduced/removed and the summary of their expression in BL21 strain of *E. coli*

Construct	Position[a]	Restriction site	Expression in BL21	
			Periplasm	Inclusion bodies
pKA-1	H266A[b, c]	+*Xba*I	—	++
pKA-2	P268A[c]	None	++	—
pKA-3	R274A[c]	+*Sac*II	++	—
pKA-4	H275A	+*Sac*II	++	—
pKA-5	R276A	+*Sac*II	++	—
pKA-6	Q277A	+*Sac*II	++	—
pKA-7	R279A	+*Xba*I	++	—
pKA-8	G280A[c]	+*Sac*II	++	—
pKA-9	W281A	+*Sac*II	—	++
pKA-10	E282A	+*Sac*II	++	—
pKA-11	E285A	+*Sac*II	++	—
pKA-12	Q286A	+*Sac*II	++	—
pKA-13	P290A[c]	None	++	—
pKA-14	R293A	+*Hin*dIII	++	—
pKA-15	R302A	−*Sal*I	++	—
pKA-16	N306A	−*Sal*I	++	—
pKA-17	Q307A[c]	−*Sal*I	++	—
pKA-18	Q310A[c]	−*Sal*I	++	—
pKA-19	I312A[c]	−*Sal*I	++	—
pKA-20	R313A[b]	−*Sal*I	—	++
pKA-21	L316A	+*Sac*II	++	—
pKA-22	P319A	+*Bam*HI	++	—
pKA-23	E327A	+*Bam*HI	++	—
pKA-24	R330A	+*Bam*HI	++	—
pKA-25	E331A	+*Sac*I	++	—
pKA-26	R352A[d]	+*Sac*II	—	—
pKA-27	T355A[b]	+*Sac*II	—	++
pKA-28	N357A	+*Sac*II	++	—
pKA-29	N364A[c]	+*Sac*II	++	—
pKA(d)-1	P268,290A[c]	None	++	—

[a] The amino acid indicated prior to the residue number is the wild type amino acid and alanine replacement is indicated after the residue number. +, introduction of a unique restriction site; −, removal of a unique restriction site; ++, good expression; —, no expression.

[b] The mutant protein was secreted in the periplasm when BL21 strain containing plasmid encoding GroEL was used for expression.

[c] Amino acids with accessible surface area <90 Å^2.

[d] The mutant protein was not secreted in the periplasm in the expression system tested.

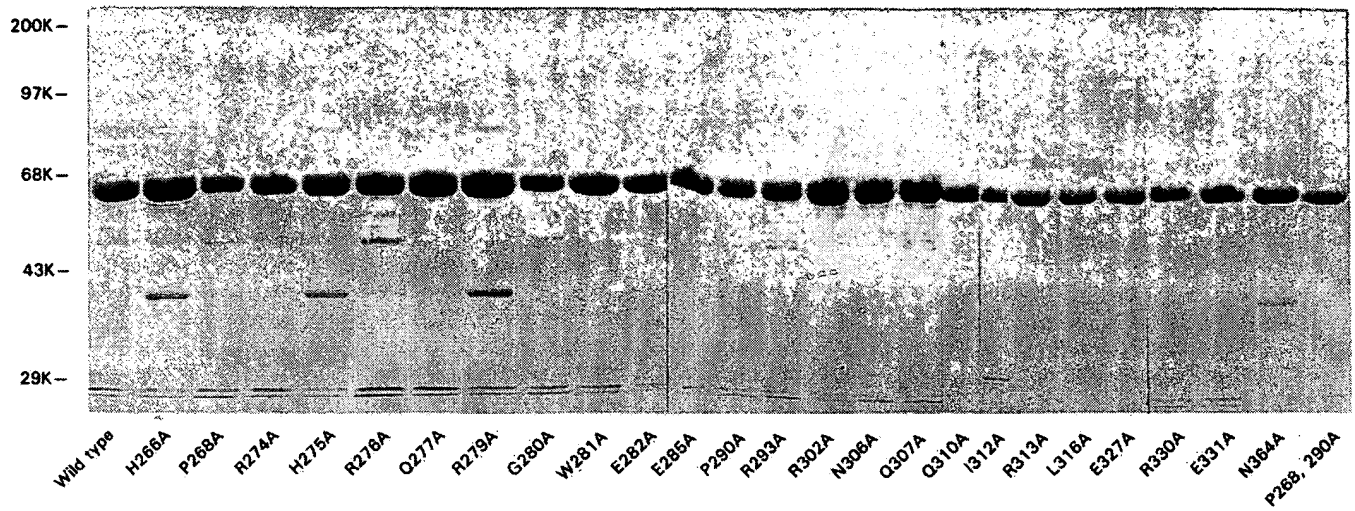

FIG. 1. **SDS-polyacrylamide gel electrophoresis of purified PE and representative mutant proteins.** The purified proteins were run on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. The gel was stained with Coomassie Blue. *Numbers* on the *left* indicate molecular weight markers in daltons.

effect of the mutations on the cytotoxic activity of PE, protein synthesis inhibition assays were performed on mouse L929 cells and on human A431 and MCF7 cells. Each protein was tested at least three times on each cell line to be certain that the cytotoxic activities obtained were reproducible. The ID_{50} values of wild type PE on the L929, A431, and MCF7 cell lines were 0.375, 2.1, and 17 ng/ml, respectively. Based on their cytotoxic activity on different cell lines, the mutants can be divided into seven categories (Table II). The first category consists of mutants H266A, R274A, R276A, R279A, W281A, R293A, and I312A; these were completely inactive on all three cell lines. The second category consists of seven mutants R302A, Q307A, R313A, L316A, R330A, P319A, and T355A; these were severalfold less active than native PE on all three cell lines. The third contains mutant E282A, which had the same activity as wild type PE on L929 cells and A431 cells but was more active on MCF7 cells. The fourth contains mutant proteins H275A, Q277A, G280A, Q286A, N306A, E327A, E331A, N357A, and N364A; these proteins were severalfold less active on L929 and A431 cells but had different activities on MCF7 cells. G280A, E331A, and N364A were just as active as PE on MCF7 cells, whereas proteins H275A, Q277A, Q286A, N306A, E327A, and N357A were more active than PE on MCF7 cells. Q310A falls into category five, because compared with PE it is slightly more active on L929 cells, less active on A431 cells, and just as active on MCF7 cells. Category six contains E285A which was slightly more active on L929 and MCF7 cell lines than PE but less active on A431 cells. Category 7 consists of a set of proline mutants which will be described separately below. The ID_{50} values of all the mutants on the three cell lines are shown in Table III. In order to make comparison of the cytotoxic activities clearer, the relative activities on different cell lines in comparison to native PE which is set at 100% is plotted in Fig. 2.

ADP-ribosylation Activity of Mutants—PE has a compact structure and as a consequence has very little ADP-ribosylation activity when assayed without urea and DTT; the presence of these substances unfolds and thereby activates the protein. As expected because the ADP-ribosylation activity resides in domain III all of the mutant proteins retained full ADP-ribosylation activity when assayed in the presence of urea and DTT. But the mutant proteins can be separated into two groups based on their ADP-ribosylation activity in the absence of urea and DTT. Five mutants H275A, E282A, E285A, Q286A, and Q310A had considerable amounts of ADP-ribosylation activity without urea and DTT. All the other mutant proteins behaved like wild type PE requiring urea and DTT to unmask ADP-ribosylation activity.

Protease Sensitivity of Mutant Proteins—Improperly folded proteins often display a change in their sensitivity to proteases. To assess the effect of these point mutations on the protease sensitivity of PE, the purified proteins (10 μg) were digested with 40,200 and 500 ng of trypsin at pH 7.4. The data is summarized in Table IV. The mutants fall into three groups. The first group includes H266A, W281A, N306A, R313A, L316A, P319A, E327A, T355A, N357A, and N364A; these are all much more sensitive to trypsin than PE. The second group includes eight mutants, G280A, E282A, E285A, R293A, R302A, Q310A, I312A, and E331A which behave like wild type PE. The third group includes R274A, H275A, R276A, Q277A, R279A, Q286A, Q307A, and R330A; these are more resistant to trypsin than native PE. Within the group of trypsin-resistant mutants, some are highly resistant (H275A, R276A, Q277A, R279A, Q286A, and R330A) and some are moderately resistant (R274A and Q307A). Fig. 3 shows the representative examples from each group of mutants. Because trypsin cleaves next to basic amino acids, increased resistance was to be expected with many of these mutants. Therefore, chymotrypsin digestion was also carried out to analyze protein structure. PE is fairly resistant to chymotrypsin action, so that the purified proteins (10 μg) were treated with 0.5, 1, and 2 μg of chymotrypsin at pH 7.4; the data are summarized in Table V. The mutant proteins fall into three different groups depending on their sensitivity to chymotrypsin. R274A, H275A, R276A, Q277A, and N3357A are more sensitive to chymotrypsin than native PE. H266A, W281A, Q286A, L316A, and T355A are most resistant than native PE. All other mutant proteins behave like wild type PE. Fig. 4 shows representative examples from each group of mutants.

Mutation of Proline Residues in Helix A and B of Domain II—Domain II of PE is predominantly composed of α-helices. Some of these helices contain proline residues which disrupt these helices. To investigate the role of prolines in the structure of PE, we replaced two of these proline residues at positions 268 and 290 by alanine. We also constructed a

TABLE II

Toxic activity of PE and all of its alanine mutants on different cell lines

Group	Position[a]	Cytotoxicity ID_{50}		
		L929	A431	MCF7
		ng/ml		
Native PE		0.38	2.1	17
1	H266A[b]	NT	NT	NT
	R274A	NT	NT	NT
	R276A	NT	NT	NT
	R279A	NT	NT	NT
	W281A	NT	NT	NT
	R293A	NT	NT	NT
	I312A	NT	NT	NT
2	R302A	0.8	15	30
	Q307A	0.6	13.5	35
	R313A[b]	1	7	30
	L316A	1	9	30
	R330A	1.5	6	24
	P319A	30	35	40
	T355A[b]	45	47	56
3	E282A	0.3	3	5
4a	G280A	0.5	11	15
	E331A	1.2	7.2	20
	N364A	0.5	11	20
4b	H275A	1.7	30	5.4
	Q277A	1.8	20	5.4
	Q286A	3.0	7	5
	N306A	1.8	30	12
	E327A	5	11	12
	N357A	47	40	14
5	Q310A	0.1	11	16
6	E285A	0.1	5	5
7[c]	P290A	0.34	1.8	15
	P268A	0.2	1.2	13
	P268,290A	0.25	1.3	13

[a] The amino acid indicated prior to the residue number is the wild type amino acid and alanine replacement is indicated after the residue number. The values of ID_{50} given are mean of two to three independent experiments. The difference in the values for L929, A431, and MCF7 cell lines are less than 0.1, 1, and 2, respectively. NT, nontoxic.

[b] Mutant protein was secreted in the periplasm when BL-21 strain containing plasmid encoding GroE was used for expression.

[c] Amino acids that are not exposed on the protein surface.

TABLE III

ADP-ribosylation activity of PE and mutant molecules in presence and absence of urea and DTT

Group	Position[a]	ADP-ribosylation activity	
		Without urea and DTT	With urea and DTT
		%	
Native PE		0	100
1	H275A	23	97
	E282A	14	91
	E285A	25	87
	Q286A	12	109
	Q310A	18	79
2	H266A	0	97
	R274A	0	86
	R276A	0	103
	Q277A	0	79
	R279A	0	81
	G280A	0	96
	W281A	0	93
	R293A	0	91
	N302A	0	110
	N306A	0	107
	Q307A	0	111
	I312A	0	102
	R313A	0	98
	L316A	0	87
	P319A	0	79
	R330A	0	93
	E331A	0	87
	T355A	0	93
	N357A	0	101
	N364A	0	89

[a] The amino acid indicated prior to the residue number is the wild type amino acid and alanine replacement is indicated after the residue number. %, relative activity in percentage. The ADP-ribosylation activity of wild type PE with urea and DTT is considered as 100%.

double mutant by replacing both P268 and P290 by alanine. P268A and the double mutant P268,290A were slightly more active than PE on all the three cell lines tested, whereas P290A was equally active as wild type PE (Table II). All the three mutant proteins showed full ADP-ribosylation activity in presence of urea and DTT. P268A did not exhibit ADP-ribosylation activity in the absence of urea and DTT whereas P290A and P268,290A showed partial ADP-ribosylation activity (9 and 13%, respectively). P268A and P290A were equal to native PE in their trypsin sensitivity whereas the double mutant P268,290A was more sensitive to trypsin. All the three mutants were identical to PE in their chymotrypsin sensitivity.

DISCUSSION

Domain II of PE has an important role in the translocation of the toxin into the cytosol (10). In the present study, we used alanine scanning mutagenesis to investigate the role of amino acids on the surface of Domain II in the cytotoxic action of PE. Unexpectedly, changes in each of the 27 surface residues that were mutated to alanine produced proteins with altered cytotoxic activity.

Inactive Mutants—Seven alanine mutants (H266A, R274A, R276A, R279A, W281A, R293A, and R313A) lost their cytotoxic potency completely. Five (H266A, R274A, R276A, R279A, and W281A) of these have mutations in a protease-sensitive arginine-rich loop. Evidence has already been provided that the full-length toxin undergoes proteolytic cleavage in this arginine-rich loop region between arginine 279 and glycine 280. This is followed by reduction of a disulfide bond spanning residues 265–287 to generate a 37-kDa carboxyl-terminal fragment that is ultimately translocated to the cytosol (10). The complete loss of activity in these five mutants may be due to the alteration of the protease recognition site due to replacement of its critical amino acids. These inactive mutant proteins would be deficient in proteolytic processing which is a key step for further action of PE. Trypsin resistance of R274A, R276A, and R279A is attributed to the replacement of a target arginine residue by alanine, and the chymotrypsin resistance of W281A is due to the replacement of chymotrypsin target tryptophan by alanine. Unexpectedly, (*a*) H266A and W281A are more sensitive to trypsin, and (*b*) H266A is more resistant and R274A and R276A are more sensitive to chymotrypsin than native PE although these mutations are not the immediate target sites of these proteases. These data indicate that changes in the conformation of PE caused by mutations H266A, R274A, R276A, and W281A may lead to defective proteolytic processing or disulfide bond reduction. In the two mutant proteins with alanine at positions 293 and

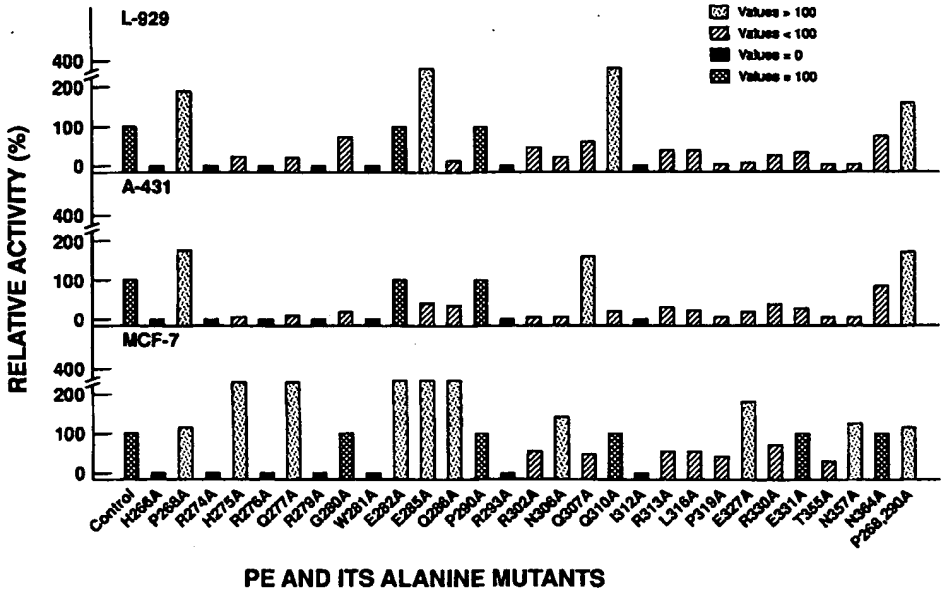

FIG. 2. **The relative cytotoxic activity of the alanine mutant proteins on three different cell lines compared with PE.** ■, inactive; ▨, less active; ▩, equally active; □, more active.

TABLE IV
Trypsin sensitivity of PE and PE mutants

Group	Position[a]	Proteolysis trypsin			
		0	40	200	500
				ng	
Native PE		–	–	++	+++
1) Trypsin-sensitive mutant	H266A	–	+++	+++	+++
	W281A	–	+++	+++	+++
	N306A	–	+++	+++	+++
	R313A	–	+++	+++	+++
	L316A	–	+++	+++	+++
	P319A	–	+++	+++	+++
	E327A	–	+++	+++	+++
	T355A	–	+++	+++	+++
	N357A	–	+++	+++	+++
	N364A	–	+++	+++	+++
2) Control (equivalent to wild type)	G280A	–	–	++	+++
	E282A	–	–	++	+++
	E285A	–	–	++	+++
	R293A	–	–	++	+++
	R302A	–	–	++	+++
	Q310A	–	–	++	+++
	I312A	–	–	++	+++
	E331A	–	–	++	+++
3) Trypsin-resistant mutants	R274A	–	–	+	+++
	Q307A	–	–	+	+++
	H275A	–	–	–	+
	R276A	–	–	–	+
	Q277A	–	–	–	+
	R279A	–	–	–	+
	Q286A	–	–	–	+
	R330A	–	–	–	+

[a] The amino acid indicated prior to the residue number is the wild type amino acid and alanine replacement is indicated after the residue number. –, resistant; +, partially sensitive; ++, sensitive; +++, highly sensitive.

313, the site of mutation is distant from the site of cleavage. In addition, these proteins appear to have a structure like that of a native protein. 1) They are secreted into periplasm and behave identically to native PE during purification; 2) they have the same ADP-ribosylation activity of PE; and 3) they behave identically to native PE in their protease sensitivities, which reflect the conformation of PE. Because the position of the mutation is distant from the site of cleavage, it is likely that these molecules are processed normally and may be defective in a step after processing such as an intracellular transport step or in translocation step. Thus, the complete loss of cytotoxic activity can be attributed to either defective proteolytic processing or a later step such as translocation.

Less Active Mutants—Seven mutants that are severalfold less active than PE on all the three cell lines tested are R302A, Q307A, R313A, L316A, P319A, R330A, and T355A. Six of these, Q307A, R313A, L316A, P319A, R330A, and T355A, showed varied protease sensitivity as shown in Tables IV-VI. Surprisingly, R313A has increased trypsin sensitivity despite replacing a trypsin-sensitive target arginine on the surface of the PE molecule by alanine. This indicates a change in the overall conformation of the molecule. Trypsin sensitivity of L316A, P319A, and T355A, trypsin and chymotrypsin resistance of Q307A and L316A, respectively, was also unexpected because leucine, proline, threonine, and glutamine are not trypsin and chymotrypsin targets. This further confirms the change in the conformation of the mutant protein which causes protease resistance to the mutants. However, the trypsin resistance of R330A can be attributed to the replacement of trypsin target arginine on the surface by alanine. This suggests the importance of these amino acids in attaining the native functional conformation of PE. R302A behaves like PE even in its protease sensitivity indicating that this mutation is not affecting the native conformation/folding but may have an important role in some stage of cytotoxic action. This mutant may be a translocation-deficient mutant. Thus, the reduction in cytotoxicity may be due to the above-mentioned possibilities like global misfolding/perturbation or deficiency in translocation.

Inactive/Less Active Mutants Defective in Secretion—Three mutant proteins, one that was inactive (H266A) and two that had low cytotoxic activity (R313A and T355A), aggregated within the bacterial cells while all other mutant proteins were secreted normally into periplasm. Formation of intracellular aggregates is probably determined by a combination of effects including the rate of folding and aggregation, the solubility and thermodynamic stability of folding intermediates, and the native state and interactions with chaperones (20). The failure of these mutant proteins to be secreted into the peri-

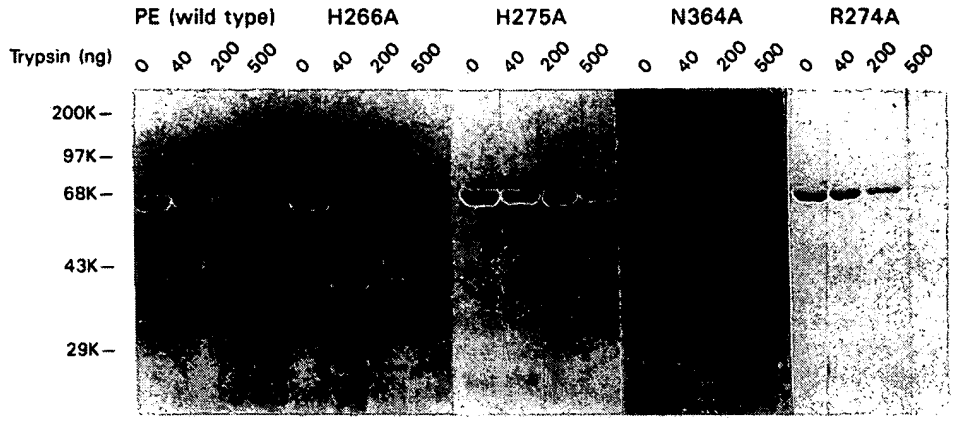

FIG. 3. **Digestion of PE and representative mutant proteins with trypsin.** Equal amounts of each protein were incubated with the indicated concentration of trypsin. Proteins were resolved on 12.5% polyacrylamide gels and stained with Coomassie Blue. *H266A* and *N364A*, trypsin sensitive; *H275A*, highly resistant; *R274A*, moderately resistant.

TABLE V

Chymotrypsin sensitivity of PE and mutant molecules

Group	Position[a]	Proteolysis chymotrypsin			
		0	500	1000	2000
				ng	
Native PE		–	+	+++	+++
1) Chymotrypsin-sensitive mutants	R274A	–	+++	+++	+++
	H275A	–	+++	+++	+++
	R276A	–	+++	+++	+++
	Q277A	–	+++	+++	+++
	N357A	–	+++	+++	+++
2) Control (equivalent to wild type)	R279A	–	+	+++	+++
	G280A	–	+	+++	+++
	E282A	–	+	+++	+++
	E289A	–	+	+++	+++
	R302A	–	+	+++	+++
	N306A	–	+	+++	+++
	Q307A	–	+	+++	+++
	Q310A	–	+	+++	+++
	I312A	–	+	+++	+++
	R313A	–	+	+++	+++
	P319A	–	+	+++	+++
	E327A	–	+	+++	+++
	R330A	–	+	+++	+++
	E331A	–	+	+++	+++
	N364A	–	+	+++	+++
3) Chymotrypsin-resistant mutants	H266A	–	–	–	+++
	W281A	–	–	+	+++
	Q286A	–	–	+	+++
	L316A	–	–	+	+++
	T355A	–	–	+	+++

[a] The amino acid indicated prior to the residue number is the wild type amino acid and alanine replacement is indicated after the residue number. –, resistance; +, partially sensitive; +++, highly sensitive.

plasm indicates that they are not capable of attaining a proper conformation prior to secretion. The influence of the GroEL system on the acquisition of structure by recombinant proteins has been demonstrated for dimeric Rubisco produced in *E. coli* (20). We expressed the mutant PE proteins that aggregated in the cytosol in a strain containing a plasmid encoding GroE, to try to prevent the proteins from aggregating by keeping them in a conformation competent for secretion. We found that a portion of all three mutant proteins appeared in the periplasm, although most remained behind within the spheroplasts. This result suggests that GroE helps the mutant protein to attain a conformation that allows for its secretion into the periplasm.

Mutants with Varied Cytotoxic Potencies on Different Cell Lines—We found several mutants that had different cytotoxic activities depending on the cell line used. Undoubtedly, the interaction of PE with mammalian cells is complex. To reach the cytosol, PE must encounter several different pH environments and many different cellular components. Cells from different species and different tissues are likely to have different relative amounts of toxin receptors, proteases, chaperones, and organelle constituents. It is therefore reasonable to expect that individual point mutations will be handled differently in different cell types.

Active Mutants—PE has a compact three-dimensional structure and has very little ADP-ribosylation activity *in vitro* unless it is unfolded by denaturing reagents like urea and DTT. This group of mutants (E282A, E285A, and E310A) which in most cases has retained full cytotoxic activity or has enhanced cytotoxic potency on some cell lines exhibits partial ADP-ribosylation activity in the absence of urea and DTT indicating that these mutant proteins may be partially unfolded. However, they differ among themselves in other properties. E282A is indistinguishable from native PE in terms of its cytotoxic potency on L929 and A431 cell lines and its protease sensitivity, but it is more toxic on the MCF7 cell line and displays some ADP-ribosylation activity without activation. This suggests that the mutant protein has a partially unfolded structure which is favorable for the full expression of cytotoxic action of PE.

Q310A and E285A showed increased cytotoxic potency on L929 cells yet decreased toxic activity on A431 cells. E285A, a mutant in arginine-rich loop region, had higher toxic activity on MCF7 cells. Both mutant proteins do not require preactivation for expression of ADP-ribosylation activity indicating that the molecule may be partially unfolded, yet they could not be distinguished from native PE in their protease sensitivity (Table VI). Their partial unfolded conformation may favor efficient processing or translocation in L929 cells but not so in A431 cells thus bringing variation in cytotoxic potency.

We also obtained a unique mutant R352A which prevented introduction of the plasmid into the BL21 (λDE3) strain indicating that the mutant protein is highly toxic to the bacteria even when expressed at low levels and kills the cell. The colonies that survived did not express any protein.

"Helix-breaking" Mutations—P290A is indistinguishable from native PE in its cytotoxic potency and protease sensitivity, but unlike PE it does not require preactivation for *in vitro* enzyme activity which indicates that this mutant protein has an altered structure. P268A cannot be distinguished from native PE in protease sensitivity and *in vitro* enzyme activity but is more cytotoxic on all the three cell lines tested. The mutation is located in an α-helix that forms one side of the arginine-rich loop region. The increase in cytotoxic activity may be due to the replacement of the helix-breaking residues

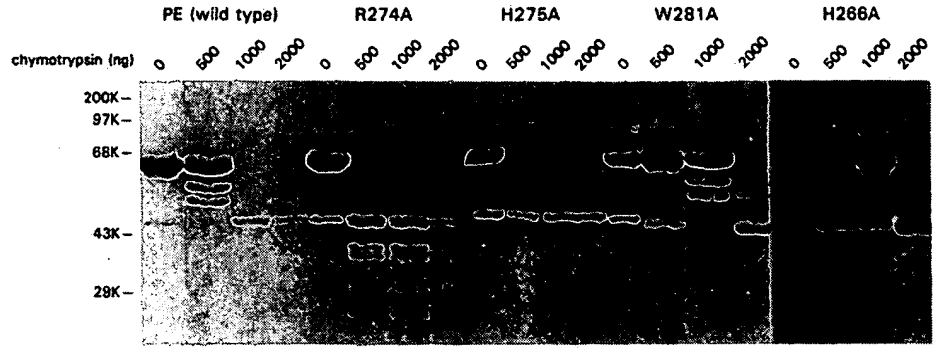

FIG. 4. **Digestion of PE and mutant proteins with chymotrypsin.** Equal amounts of each protein were incubated with the indicated concentration of chymotrypsin. Proteins were resolved on 12.5% polyacrylamide gels and stained with Coomassie Blue. *R274A* and *H275A*, chymotrypsin sensitive; *W281A* and *H266A*, chymotrypsin-resistant.

TABLE VI

Summary of properties of PE and mutant proteins

A, activity equivalent to PE; IA, inactive; LA, less active; MA, more active; PA, partially active; C, control (similar to wild type PE in protease sensitivity); S, sensitive; MR, moderately resistant; R, resistant; HR, highly resistant.

Position[a]	Cytotoxicity			ADP-ribosylation activity[b]	Protease sensitivity	
	L929	A431	MCF7		Trypsin	Chymotrypsin
Native	A	A	A	IA	C	C
H266A[c]	IA	IA	IA	IA	S	HR
R274A	IA	IA	IA	IA	MR	S
R276A	IA	IA	IA	IA	HR	S
R279A	IA	IA	IA	IA	HR	C
W281A	IA	IA	IA	IA	S	R
R293A	IA	IA	IA	IA	C	C
I312A	IA	IA	IA	IA	C	C
R302A	LA	LA	LA	IA	C	C
Q307A	LA	LA	LA	IA	MR	C
R313A[c]	LA	LA	LA	IA	S	C
L316A	LA	LA	LA	IA	S	R
P319A	LA	LA	LA	IA	S	C
T355A[c]	LA	LA	LA	IA	S	R
R330A	LA	LA	LA	IA	HR	C
G280A	LA	LA	A	IA	C	C
E331A	LA	LA	A	IA	C	C
N364A	LA	LA	A	IA	S	C
H275A	LA	LA	MA	PA	HR	S
Q277A	LA	LA	MA	IA	HR	S
Q286A	LA	LA	MA	PA	MR	R
N306A	LA	LA	MA	IA	S	C
E327A	LA	LA	MA	IA	S	C
N354A	LA	LA	MA	IA	S	S
E282A	A	A	MA	PA	C	C
Q310A	MA	LA	A	PA	C	C
E285A	MA	LA	MA	PA	C	C
P290A[d]	A	A	A	PA	C	C
P268A[d]	MA	MA	MA	IA	C	C
P268,290A[d]	MA	MA	MA	PA	S	C

[a] The amino acid indicated prior to the residue number is the wild type amino acid and alanine replacement is indicated after the residue number.

[b] ADP-ribosylation activity indicated are in the absence of urea and DTT.

[c] Mutant protein was secreted in the periplasm when BL-21 strain containing plasmid encoding GroE was used for expression.

[d] Amino acids that are unexposed.

[e] Alanine mutant defective in secretion.

proline by alanine which places the arginine residues in a conformation that is more easily cleaved by the protease that processes PE. P268,290A, the double mutant, is slightly more toxic than native PE, is more trypsin sensitive than native PE, and displays ADP-ribosylation activity without urea and DTT which suggests the toxin is partially unfolded. This favors efficient proteolytic processing or disulfide bond reduction.

All the mutants that do not require preactivation with urea and DTT to express their ADP-ribosylation activity showed an increase in cytotoxic potency on at least one cell line. This suggests that these molecules are unfolded in such a way that there is an increase in the efficiency of processing or some other step in translocation process.

In summary, alanine scanning mutagenesis identified many surface amino acids in domain II of PE that are important for its cytotoxic action, maintenance of native conformation, proper folding, secretion into the periplasm, and efficient proteolytic processing.

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